**New expression cassette for expression of arbitrary genes in plant seeds**

**Description**

The invention in question relates to an expression cassette for expression of arbitrary genes in plant seeds and the plasmids containing the expression cassette. The invention also includes the production of transgenic plant cells containing this expression cassette as well as the use of the plasmids in this expression cassette for production of transgenic plants. Fields of application of the invention are biotechnology, pharmacy and plant production.

For a long time now, there have been methods making it possible to integrate relevant genes into the genome of higher plants. The objective of this work is the production of plants with new properties, for example to increase agricultural production, to optimise manufacture of foodstuffs and to produce specific pharmaceuticals and other interesting ingredients. One prerequisite for the expression of the transferred genes in this context is that they possess plant-specific promoter sequences. For this purpose, so-called constitutive promoters such as the promoter of the nopaline synthase gene /1/, the TR double promoter /2/ or the promoter of the 35S transcript of the cauliflower mosaic virus /3/ are used. One disadvantage of these promoters is that they are active in almost all the tissues of the manipulated plants. In this way, a controlled and purposeful expression of the foreign genes in the plants is not possible. It is better to use promoters which function tissue-specifically and independently of development. Genes with the matching promoters, which are only active in anthera, ovaries, blooms, leaves, deciduous leaves, stems, roots or seeds, have been isolated /4/. But they differ greatly in the strength and specificity of the expression and only have a limited use. For the use of the seeds as a source of nutrition and for production of in-

gredients, it is above all the seed-specific promoters which are of great interest. With the years of research into the genes of the seed-storage proteins, some more or less specific promoters with differing strengths, for example that of phaseolin /5/ or legumin and USP /6/ are available. As these storage proteins are synthesised by gene families, fusions of such promoters with foreign genes are in competition with the endogenous numerous genes of the corresponding gene family. For this reason, it is more favourable to use promoters from unique, strongly and specifically expressing genes. For co- and multiple transformations, the use of differing regulatory sequences is suitable, in order to make better use of the development of the seed in time, to synthesise identical or differing gene products in parallel and to avoid co-suppression.

Although a number of expression cassettes for expression of arbitrary genes in plant seeds are already known, the expression rates in plant seeds achieved have not been optimal up to now for the substantiation of a plant biotechnological production of the required materials.

Ins a1

The invention therefore has the objective of placing the seed-specific expression in transgenic plants on a basis suitable for a production of materials. It is based on the task of constructing an expression cassette with which a stable expression with a high expression rate of genes of the materials to be produced can be achieved in plant seeds.

The objective of the invention is achieved with the expression cassette described in claim 1, with sub-claims 2-7 being preferred variants.

Ins a2

The expression cassette according to the invention contains the following essential component parts:

- the promoter of the gene of the sucrose binding protein (SBP)like protein

- if applicable, the DNA sequence of a signal peptide, preferably the SBP signal peptide
- a gene to be expressed
- 3' termination sequences

The invention relates above all to a regulatory DNA sequence occurring uniquely in the genome, which mediates a strong expression of an arbitrary heterologous gene primarily in the cotyledons and in the endosperm dependency on seed development.

The most important component part of the cassette is the SBP promoter, the sequence of which is shown in Figure 1. Compared with analog promoters in this field, this promoter has the benefit of great strength and seed-specificity. Its use for the expression of foreign genes, even without the DNA sequence of a signal peptide, is also part of the scope of the invention.

Together with the transcriptionally regulatory sequences, the expression cassette also, if need be, contains a signal peptide, which enables the transport of the required gene product into the protein bodies, thus preventing decomposition of the gene products to a great extent. The optional use of the authentic signal peptide enables the transport of the synthesised foreign proteins to and storage in the protein bodies.

The genes to be expressed can be integrated either as transcription or as translation fusions, they can be varied to a great extent, for example genes can be used for the production of enzymes (e.g. amylase, xylanase), pharmaceutical products or for the over-expression of proteins with a high share of essential amino-acids (e.g. 2S globulin of the brazil nut rich in methionine) or of other proteins influencing the properties of the seeds. Further possibilities can be found in the reduction or elimination of gene products through the integration of genes in an anti-sense orientation. By inserting regulatory genes under the control of this seed-specific promoter, metabolic processes in the seeds can

also be influenced. The cassette can also be used in order to express the SBP gene inherent to the promoter from field beans into other species. The use of other terminators, for example the termination sequence of the gene to be expressed, is a further possibility of optimal use of the cassette. As a concrete example, the gene of ß-glucuronidase (GUS) was used to show the specificity of the promoter (Fig. 2b, c).

The nucleotide sequence of the expression cassette contains transcriptionally regulatory areas, guaranteeing a strong specific expression of an arbitrary gene into the seed of plants. The Northern (Fig. 2a) shows the high seed-specific expression in the various tissues of Vicia faba. The GUS data in Figs. 2b and 2c show on the one hand the distribution of the ß-glucuronidase in the sections through ripe tobacco seeds and, on the other, the accumulation of the ß-glucuronidase in the transgenic tobacco seeds as a function of development.

The plasmids containing the expression cassette, preferably the plasmids pSBPROCS and pPTVSBPRGUS, are also to be placed under protection.

The scope of the invention also includes the use of the expression cassette according to claims 12-16, which is done by transformation into bacteria strains and subsequent transfer of the resulting recombinant clones into preferably dicotyl plants. The plants expressing the required gene product in the seed are selected and bred as genetically stable lines. After harvesting, the required gene products are extracted from the transgenic seeds in a way basically already known.

This invention is also interesting for applications in which the required gene product is expressed under the control of various promoters, in order to increase the total of the expression rates, in order to make better use of the development period of the seeds and to avoid effects by co-suppression. This expression cassette is also suited for co- and

multiple transformations with the objective of expressing various gene products. A variety of new expression cassettes is needed for these strategies in order to be able to select the correct ones.

The entire method for the alteration of a plant cell is portrayed in an example (pSBPOCS).

The invention is to be explained in more detail below with examples of embodiments.

Methods

1. Cloning method

For cloning, the vectors pUC18 /7/, pBK-CMV (Stratagene) and pOCS1 (Plant Genetic Systems, Gent, Belgium) and for plant transformation the vectors BIN19 /8/, and, after deletion of the GUS gene, pGPTV-BAR /9/ were used.

2. Bacteria strains

For the transformation to E. coli, strain DH5α /10/ was used. The binary plasmids were inserted into the agro-bacteria strain EHA105 /11/ by conjugation.

3. Plant transformation

The transformation of Nicotiana tabacum was done by the leafdisk method /12/ and the transformation of Vicia narbonensis with the help of the method described by Pickardt in 1991 /13/ by agrobacterium mediated gene transfer.

4. Analysis of genomic DNA from transgenic plants

The genomic DNA of the transgenic tobaco and V. narbonensis plants was isolated with the help of the DNA isolation kit of the firm of Macherey & Nagel. In a first step, the transgenic lines were identified via PCR with gene-specific primers. The integration of foreign DNA was examined by means of "Southern

blot" analyses of 20µg of DNA following suitable restriction digestion.

5. ß-glucuronidase activity test (GUS assay)

The reporter gene ß-glucuronidase is a bacterial enzyme accessible to both quantitative /14/ and also histo-chemical activity assays. Tissue samples were incubated over night at 37°C in 1 mM X-Gluc, 50mM Na phosphate (pH 7.0) and 0.1% Tween 20. For sections, the tissues were fixed, embedded in paraffin and cut to a section thickness of 15 - 30 µm on a microtome.

Examples of embodiments

The invention, which contains the production of a new, seed-specific expression cassette as well as the plasmids and transgenic plants derived from them, is explained below - partly with the help of the figures - using an example of an embodiment.

1.) Cloning and structure analysis of an SBP seed protein gene from Vicia faba

Primers (5'-GAAGACCCTGAGCTCGTAACTTGCAA-ACAC- 3' and 5'-AGTACTCATAGATCTCTGGGTGATGTTGGT-3') were derived from the sequence of a cDNA clone which codes for the sucrose binding protein of the soybean /15/. The gene-specific probe was then amplified, cloned and sequenced by means of RT - PCR on mRNA, isolated from immature cotyledons of V. faba. The PCR product was identified as the gene fragment homologous to the sucrose binding protein and was used as a probe for the isolation of the complete cDNA from a cotyledon-specific λ Zap Express cDNA Bank of V. faba L. var. minor. One of the isolated clones (VfSBP20), which has a homology of 68% on the nucleotide level, codes for the complete SBP-homologous gene from the field bean. But it differs from the gene isolated from the soybean in both the expression (Fig. 2a) and also in the function (no sucrose binding).

2) Isolation of the regulatory sequences by means of PCR

The regulatory sequences were isolated with the help of the "Universal GenomeWalker™Kit" of the firm of CLONTECH and the gene-specific primers PSBP1, position 159 (5'-AATCCTCA-CACTTCTCCATGCATATCCGTTTGTCC-3'), PSBP2, position 118 (5'-GCCCTGCAGAT-CGCATTTGTCTTTGCA-3') and PSBP3, position 85 (5'-CTGGGTCCTTTTCTTTTCTGG- C-3'). Following prior digestion of the genomic DNA of V.faba with ScaI (a) and StuI (b) and ligation of the adapters, a two-step PCR was done in accordance with the description of the kit with the following parameters: 7 cycles of 94°C, 2s, 72°C, 3 min and 32 cycles of 94°C, 2s, 67°C, 3 min and 4 min 67°C. The PCR preparations were diluted 1:50 and 1µl of each were amplified in a second PCR (5 cycles of 94°C, 2s, 72°C, 3 min and 20 cycles of 94°C, 2s, 67°C and 4 min at 67°C. In the Agarosegel, bands of 1.7 kb from (a) and 1.9 kb from (b) were verified via a Southern blot. These bands were then cloned into the pUC18 and sequenced. The clones SBPR7 and SBPR15 were then identified by a sequence comparison as the promoters matching gene VfSBP20. They represent allelic variants of gene VfSBP20, with both clones having 100% sequence identity with clone VfSBP20 in the corresponding area. On the 5' side of the ATG of the SBP gene, 1539 bp were isolated with clone SBPR7 and 1750 bp with clone SBPR15. They differ by 23 base pair substitutions and two insertions. The restriction maps of clone pSBPR7 and pSBPR15 are shown in Fig. 3, the sequence of clone pSBPR15 in Fig. 1.

3a) Proof of the seed-specific expression in tobacco

With the help of the reporter gene of β-glucuronidase, the seed-specific expression of the isolated regulatory sequences SBPR7 and SBPR15 was to be tested. For this, the binary plasmid pBI101 /14/, which contains the promoter-free glucuronidase gene behind a poly-linker, was cut with SmaI and dephosphorylated. The promoters were isolated from the plasmids pSBPR7 and pSBPR15 respectively by means of an SalI/NcoI

digestion and the ends smoothed. The fragments were then cloned into the SmaI site of binary plasmids pBI101 in front of the reporter gene, with plasmids pBISBPR7GUS and pBISBPR15GUS resulting. These plasmids were then transferred to the agro-bacteria strain EHA105 and the chimerical agro-bacteria containing SBP promoter/glucuronidase gene were used for the transformation of tobacco. The results are shown in Figures 2b and 2c. The analysis of the transgenic tobacco seeds shows a strong blue coloration and thus a strong activity of the glucuronidase in the endosperm and in the cotyledons of the tobacco seeds, also according to the seed development. No glucuronidase activity was detected in other tissues. The two slightly different nucleotide sequences SBPR7 and SBPR15 also do not differ in their expression behaviour. These data show that the isolated regulatory sequences fused with the β-glucuronidase gene result in a strong and strictly seed-specific expression in the tobacco.

3b) Proof of the seed-specific expression in peas

In order to show that a seed-specific expression is also to be expected in legumes, the SalI/NcoI fragment of plasmid pSBPR15 was cloned into the SalI/NcoI cut plasmid pGUS1 (Plant Genetic Systems, Gent). From the resulting plasmid pSBPGUS, the fusion of the SBPR15 promoter/GUS/ocs-terminator was cut out with SalI/SmaI, smoothed and ligated into the binary plasmid pGPTV-Bar, EcoRI/SmaI cut (Fig. 4). pGPTV-Bar /9/ is a binary plasmid mediating phosphinothricin resistance which is successfully used for the transformation of peas. This plasmid has been called pPTVSBPRGUS (Fig. 4). The embryos of the transgenic pea lines generated with this plasmid show a strong blue coloration after a histo-chemical analysis.

3c) Proof of the transient expression in embryos of Vicia faba, Vicia narbonensis, Pisum sativum and Brassica napus

With plasmid pSBPGUS, isolated embryos of Vicia faba, Vicia narbonensis, Pisum sativum and Brassica napus were shot by

means of the Biolistics PDS-1000/He Particle Delivery System under the following conditions. The coating preparation comprised 50µl of gold (Hereaus, 0.6-3µm, 50 mg/ml), 10µl of Qiagen-cleaned plasmid-DNA (1µg/µl), 50µl of 2.5M $CaCl_2$ and 10µl of 0.1M spermidine. At 1800 Psi and a vacuum of 27 inch Hg, the embryos lying on an agar panel were then shot and subsequently cultivated in MS-2% sucrose liquid medium for 2 days. There was then a reaction over night at 37°C with X-Gluc (1mM) in 50mM Na phosphate (pH 7.0) and 0.1% Tween 20. Unlike the negative control (promoter-free pGUS1), a number of blue dots were registered in the above mentioned embryos, showing that the SBP promoter functions in the seeds.

4.) Production of the expression cassette for over-expression of heterologous genes in the seed

In order to make the regulatory sequences available for the over-expression of foreign genes, the SalI fragment of the longer clone SBPR15 was isolated and smoothed and cloned into the SmaI location of plasmid pOCS1 (Plant Genetic Systems, Gent, Belgium). This cassette thus contains the promoter region, the complete 5' untranslated region, the complete signal peptide, the first five triplets of the ripe protein (Fig. 1) and the 3' untranslated area with the polyadenylation signals of the octopine synthase gene (Fig. 5). The NcoI location can be used for transcription fusions with foreign genes, the BamHI location for translation fusions. After the insertion of the foreign gene, the sequence containing the promoter, regulatory sequences, the foreign gene and the 3' termination sequences is cut out with restriction enzymes and cloned into a binary vector with the herbicide resistance suitable for the plant transformation.

As an example of this, the BamHI fragment of the gene of XylanaseZ of Clostridium thermocellum was cloned into the BamHI location of plasmid pSBPOCS as a translation fusion. From the resulting plasmid pSBPRXYNZ (Fig. 6), the smoothed Asp718/SphI fragment was ligated with the binary vector

pGPTV-Bar, which was cut with the enzymes EcoRI/SmaI and smoothed. After transformation into the agro-bacteria strain EHA105, N. Tabacum was transformed. The strong expression of the Xylanase Z was shown in the ripe transgenic seeds in a Western blot (Fig. 7).

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